

On the Possibility of Automated Scoring of Pollen Mutants

by Daniel Pinkel*

Both flow cytometry and automated image analysis techniques may be useful in relieving the drudgery, increasing the speed, and adding quantitation to the scoring of pollen mutants, but practical problems related to the detection of very rare events have to be overcome. The features of flow and image cytometry instrumentation that may be useful for pollen measurements are discussed and a qualitative framework is presented from which to view the rare event problem.

Techniques such as flow cytometry and automatic image analysis may be able to reduce the drudgery and increase the speed of scoring rare pollen mutants and can quantitate the color and brightness changes that are now qualitatively determined by eye. However, because of the time and money involved in developing an automated technique, it is important to keep in mind the place such a system might take in a particular sample analysis procedure in order to judge what type and degree of automation will be most beneficial. There may be no worthwhile advantage from automation; or what might be appropriate for a central analysis facility serving many investigators might be too expensive and cumbersome for use in an individual laboratory. In the following sections I will discuss the basic features of flow cytometers and image analysis systems that may be useful in applications involving the scoring of pollen mutants and the potential sources of difficulty to be expected in detecting rare events (mutants).

Instrumentation

Flow Cytometry

In a typical flow cytometer a monodisperse suspension of the particles is made to flow single file through a region where measurements are made on each particle as it passes. A schematic diagram of

such a device is shown in Figure 1. The particle suspension is released from a small tube upstream from the nozzle in the center of a flow of liquid (usually water or saline) called the sheath. The flow of the sheath liquid is laminar so that the sample stream is reduced in diameter as it traverses the nozzle. The flow trajectories can be controlled well enough so that the particle centers all pass within a few μm of the average path. In fact the hydrodynamic forces are such that elongated objects tend to be oriented with their long axis parallel to the flow direction. Typical flow velocity in the narrow, high velocity, region of the nozzle where analysis occurs is on the order of ten meters per second. If the particles are passing at the rate of 1000/sec, they will be separated on the average by 1 cm, or about 100 particle diameters for a 100 μm diameter. At this rate it takes 17 min to measure 10^6 particles; considerably higher analysis rates are possible.

Figure 1 shows the sample particles being illuminated by a light source and the optical response of the particles being measured by detectors viewing from various directions. In many current applications the particles are fluorescently stained so that fluorescent brightness as well as light scatter is measured. With pollen it may be appropriate to measure the extinction of light in the incident beam, as well as light scattered to the side.

The time course of the electrical output of the optical detector due to the passage of a single particle is schematically illustrated in the upper right of the figure. Several characteristics of the pulse that can be measured are illustrated. The two most commonly used are the pulse height and pulse area, but I want to emphasize here the additional possibility of pulse length because it may be useful

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in discriminating signals from particles that are the wrong size to be pollen grains, and thus allow rejection of spurious objects.

The output of the instrument is a histogram, schematically pictured in the lower right, of the value of the measurement versus the numbers of objects with that value. Multiple simultaneous measurements on each object can be used to increase the discrimination, in principle generating histograms of higher dimensionality.

Image Cytometry

Image analysis instruments appropriate for pollen measurements consist basically of an optical system (microscope) for acquiring the image, a television type camera for converting the image to electrical signals, a computer for processing the camera output, and a method of scanning the sample. Because the sample is usually fixed to a slide or other substrate, the positions of the indi-

vidual pollen grains are well defined. Thus objects of particular interest can be relocated for more detailed measurements, and if desired presented to a human observer for examination. The analysis rate of an image processing instrument depends on the number of particles in each field of view, the time necessary to acquire and extract the information in the image of a field, and the time necessary to move to the next field. A major factor here is the complexity of the analysis that is desired. If one wants only to count and record the positions of a few objects that are darker (or lighter) than a certain threshold level, the cost is low both in terms of money and time (automated bacterial colony counters do this routinely); if it is necessary to do shape determinations of each particle to reject debris and to discern whether a large object is really several pollen grains in contact with each other, the cost and effort are much higher. In some cases adjusting the sample preparation procedure so that it is appropriate for a simpler instrument is

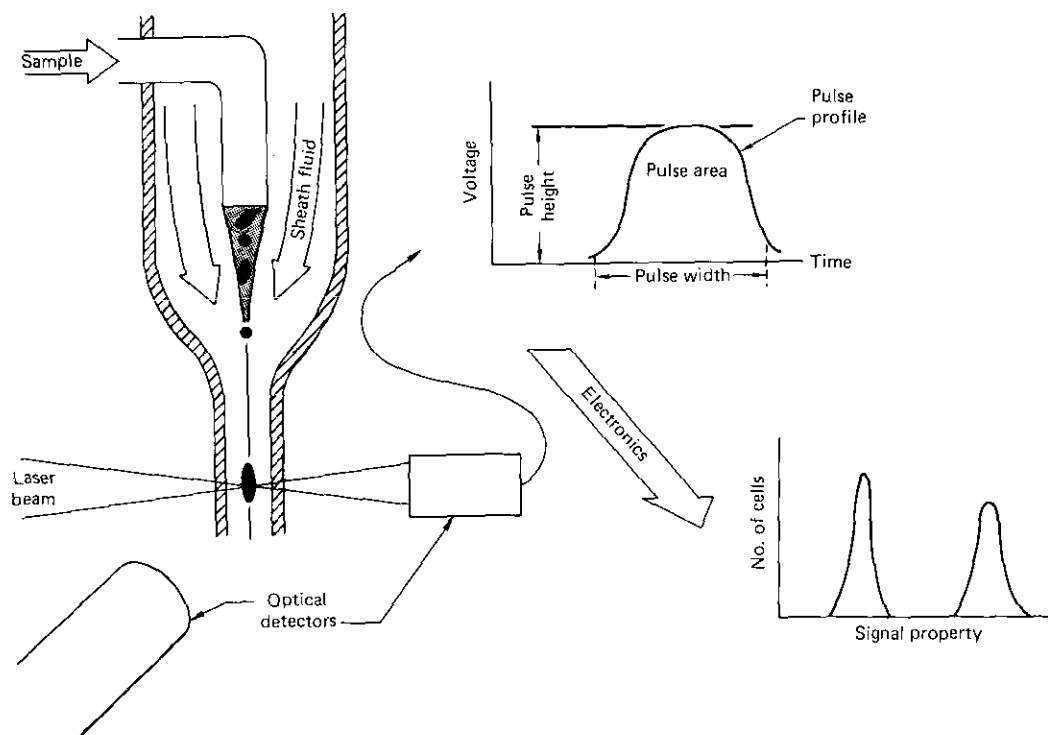


FIGURE 1. Flow cytometry. A solution containing a suspension of the sample particles is injected into the center of a nozzle containing a flow of sheath liquid. In the narrow region of the nozzle, the particles, traveling in single file and well separated, intersect some source of illumination. Detectors looking at the intersection point pick up an optical signal (fluorescence, scatter, extinction, etc.) from the passage of a particle and convert it to an electrical signal. The graph of the time course of this electrical pulse in the upper right depicts some signal properties that can be determined and used to classify each particle. A distribution of a particular one of these properties for the sample being measured is shown in the lower right. Note that the two peaks correspond to the two different types of particles depicted in the sample.

more effective than building a machine that can handle difficult samples.

The Rare Event Problem

Although the requirements of individual pollen systems vary, in general there appear to be four classes that need to be distinguished: (1) normal pollen grains, (2) aborted pollen grains, (3) mutant pollen grains, (4) debris. These discriminations present different problems. Measuring the total number of pollen grains with sufficient accuracy is not difficult because the experimental results (mutant frequencies) are not very sensitive to this parameter. Thus the misclassification of some debris as pollen may be tolerable. Counting the aborted pollen grains, if necessary, may present problems, depending on how sophisticated an analysis has to be done to discriminate them from normals and debris. It may be possible and advantageous to physically separate the abortions on a gradient of some sort so they can be counted separately from the rest of the sample. Without having attempted these measurements it is difficult to say more. However, there are some general comments that may be useful in guiding thought about the detection of mutants, that is the problem of rare events.

Mutant pollen grains occur at a frequency on the order of 10^{-4} to 10^{-6} . Assuming 10^{-5} as a representative number, classification errors concerning the mutants must be much less than one in 10^5 for any analysis scheme to be useful. It is difficult to appreciate the size of this number. For example, there are about 10^5 seconds in one day. Thus if you examined pollen grains at the rate of one per second, on the average a true mutant would be detected every 24 hr. For useful results, the time between classification errors would have to be on the order of a week. Viewed another way, 10^5 maize pollen grains placed single file in contact with each other would form a line 10 m long (for 100 μ m diameter grains). In that line, on the average, would be one mutant. The distance between errors in classification would have to be on the order of a length of a football field for a practical system.

With pollen measurements we can anticipate errors from (at least) two sources. The first are due to non-pollen debris in the sample, and to deal with these involves an appropriate combination of instrumental and sample preparation sophistication. For example, objects that are too large or small can be recognized easily in flow by using the pulse width measurement and can be ignored.

The second source of confusion comes from the

variability in darkness of the stained pollen and the necessity for establishing a criteria for defining which cells are "mutant." The number of times a human scorer does a "double take" and has to spend extra time deciding the classification of a particular grain gives some feeling for this problem. The general point here is that machines give graded, quantitative answers to questions that are now answered on a yes or no basis, and people will have to deal appropriately with this additional information. On the positive side, this ability to perhaps quantitate the degree of expression of a mutation may be a useful contribution of instrumentation even in situations where there is no advantage in speed. Clearly in this situation adequate standardization of staining is important.

These problems can be illustrated by considering the situation with corn pollen mutations. Here human observers make discriminations of the mutants by judging the darkness of the staining (Note that the darkness may not be related to stain content in a straightforward way, since the starch granules in the pollen do considerable scattering. Thus, there may not be a well defined light path over which to measure absorbance. As a recognition of this fact, people do not use transmitted light for visual scoring, but employ some combination of transmitted and reflected illumination). Imagine an automated system, either image or flow, set up to do this same "relative darkness" determination. The hypothetical output of such an instrument, shown in Figure 2, consists of a histogram of the number of objects with a given darkness versus the measured darkness. Some of these objects are not pollen. The distribution has a large peak, representing about 10^6 objects, and a tail extending to increasing darkness. The region where the darkly stained mutants are expected is circled and shown in more detail for two possible outcomes, one pessimistic and the other optimistic. Each square here represents the measurement on a single object; those due to pollen grains are black, those due to other artifacts (debris) are white in the illustration, but in real data no such distinction is visible. The pessimistic picture shows a lot of artifacts along with the pollen grains and a continuous distribution in darkness for the pollen. One here is faced with the two fundamental problems; setting the threshold for the mutants and having the measurement swamped by the debris. It may be possible to find a more appropriate measurement technique that results in the optimistic picture. Here the number of artifacts has been sufficiently reduced and the mutant pollen grains appear clearly separated from the normal ones while still showing some variability. Unfortunately this discussion can-

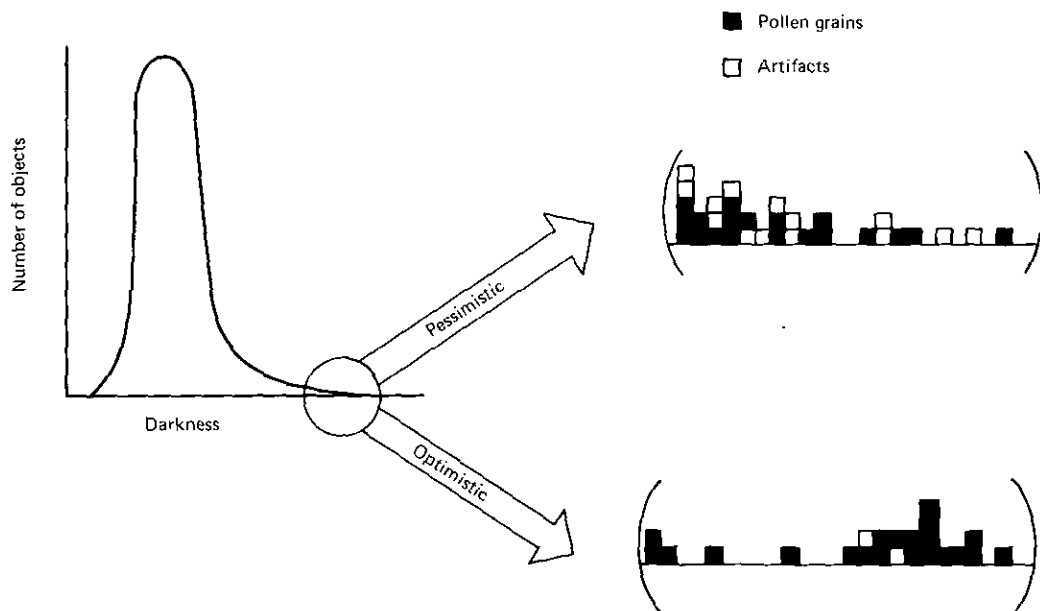


FIGURE 2. The rare event problem. The main peak of the distribution contains measurements on about 10^6 normal pollen grains and some debris. Mutants are imagined to be darker and thus expected at the right side of the peak. The pessimistic detailed view of this region shows a continuous distribution of darkness for the measurements on pollen (black squares) and the presence of many artifactual signals. In real data one could not distinguish the signals due to pollen from those due to debris so even if a rational threshold for mutants could be established the biological response of the samples would be obscured. In the optimistic view, the detection scheme has been improved to both reject the debris more efficiently and enhance the discrimination of dark pollen. A population of very dark grains (mutants) can now be clearly distinguished.

not be made more concrete until measurements are made on real samples under carefully controlled conditions to get a feeling for the actual difficulties, but I hope it serves to give a framework with which to think about the problem.

Conclusions

Both flow and image analysis machines may be helpful for scoring of pollen mutants, but the practical problems that have been discussed must be evaluated in order to allow an intelligent choice

of how, or whether, to proceed in a particular application. This requires measurements on actual samples with various trial detection schemes tailored to the problem at hand. Out of the creative tension between "that dumb machine" and "those awful samples" a useful total analysis procedure can often be developed by exploiting the important biological and physical technology.

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